

AR

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|--|--|---|--|
| (51) International Patent Classification ⁷ : C12Q 1/68, A01K 67/027 | | A2 | (11) International Publication Number: WO 00/66787 (43) International Publication Date: 9 November 2000 (09.11.00) |
| (21) International Application Number: PCT/US00/12366 (22) International Filing Date: 5 May 2000 (05.05.00) | | (81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). | |
| (30) Priority Data: 60/132,663 5 May 1999 (05.05.99) | | US | Published <i>Without international search report and to be republished upon receipt of that report.</i> |
| (71) Applicant (<i>for all designated States except US</i>): OHIO UNIVERSITY [US/US]; Suite 190, 20 East Circle Drive, Athens, OH (US). | | | |
| (72) Inventors; and (75) Inventors/Applicants (<i>for US only</i>): KOPCHICK, John, Joseph [US/US]; 4 Orchard Lane, Athens, OH 45701 (US). TIONG, Jean [US/US]; 320 W. Union Street, Apt. 215, Athens, OH 47501 (US). | | | |
| (74) Agent: COOPER, Iver, P.; Browdy and Neimark, P.L.L.C., Suite 300, 624 Ninth Street N.W., Washington, DC 20001-5303 (US). | | | |
| (54) Title: GROWTH HORMONE-REGULATABLE LIVER GENES AND PROTEINS, AND USES THEREOF | | | |
| (57) Abstract Growth hormone-regulatable liver genes and proteins are described. These may be used as diagnostic markers of liver pathology. | | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|----|--------------------------|----|--|----|--|----|--------------------------|
| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
| AM | Armenia | FI | Finland | LT | Lithuania | SK | Slovakia |
| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | ML | Mali | TR | Turkey |
| BG | Bulgaria | HU | Hungary | MN | Mongolia | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MR | Mauritania | UA | Ukraine |
| BR | Brazil | IL | Israel | MW | Malawi | UG | Uganda |
| BY | Belarus | IS | Iceland | MX | Mexico | US | United States of America |
| CA | Canada | IT | Italy | NE | Niger | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NL | Netherlands | VN | Viet Nam |
| CG | Congo | KE | Kenya | NO | Norway | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NZ | New Zealand | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | PL | Poland | | |
| CM | Cameroon | KR | Republic of Korea | PT | Portugal | | |
| CN | China | KZ | Kazakhstan | RO | Romania | | |
| CU | Cuba | LC | Saint Lucia | RU | Russian Federation | | |
| CZ | Czech Republic | LI | Liechtenstein | SD | Sudan | | |
| DE | Germany | LK | Sri Lanka | SE | Sweden | | |
| DK | Denmark | LR | Liberia | SG | Singapore | | |
| EE | Estonia | | | | | | |

**GROWTH HORMONE-REGULATABLE LIVER GENES AND
PROTEINS, AND USES THEREOF**

BACKGROUND OF THE INVENTION

Field of the Invention

5 This invention relates to the diagnosis of abnormal GH activity or general pathological activity in the liver.

Description of the Background Art

Growth Hormones:

10 The growth hormones are vertebrate proteins with about 191 amino acid residues, the number varying from species to species. There are four cysteine residues, and two disulfide bridges. The 3D-structure of porcine GH is known; it is composed of four major antiparallel alpha-helices, at residues 7-34, 75-87, 106-127 and 152-183.

15 The 3D structure of the hGH:hGH receptor complex is also known. Each molecule of hGH binds two molecules of the receptor. hGH binds to two binding sites on hGH receptor. Helix 4, the loop residues 54-74, and, to a lesser extent, helix 1, mediate binding to binding site 1. Helix 3
20 mediates binding to binding site 2.

See generally Harvey, et al., Growth Hormone (CRC Press: 1995).

25 GH is synthesized and secreted by the somatotrophic and somatomammotrophic cells of the lateral anterior pituitary. The control of GH production and secretion is complex, but is mainly under the influence of growth hormone releasing hormone (GHRH) and somatostatin, which stimulate and inhibit it, respectively. The shifting balance between these regulatory agents is responsible for the pulsatile nature of
30 GH secretion, with normal human concentrations ranging from a baseline value < 1 µg/L to peaks of 25-50 µg/L. Glucocorticoids and thyroid hormones, and various carbohydrates, amino acids, fatty acids and other biomolecules, are also known to directly or indirectly
35 regulate GH secretion.

Most GH is secreted at night, during deep sleep, but some is secreted in response to exercise and other forms of

physical stress. About 500 µg/m² body surface area are secreted by women, and 350 by men. GH secretion rates are highest in adolescents and lowest in the elderly. GH has a plasma half life of about 20-25 min. and is cleared at a 5 rate of 100-150 ml/m² body surface area.

Metabolic and Clinical Effects of Growth Hormone:

Chronic elevation of growth hormone levels in humans usually results in either gigantism or acromegaly. GH, besides affecting skeletal growth, can also influence other 10 organ systems, in particular, the liver and kidney. In the kidney, it has been associated with glomerulosclerosis and nephropathy. (Diabetic glomerulosclerosis and nephropathy has been attributed to a GH effect.) In the liver, it has been shown to cause an increase in liver size, as a consequence 15 of both hyperplasia and hepatocyte hypertrophy. The hepatocellular lesions associated with high GH levels progress with age. See Quaife, et al, Endocrinol., 124: 49 (1989); Sharp, et al., Lab. Anim. Sci., 45:607-612 (1995).

There is reason to believe that excessive GH activity 20 in the liver is deleterious to health. Mice that express GH transgenes typically live to only about one year of age, while the normal life expectancy for mice is 2-2.5 years. A major cause of death in the GH transgenic mice has been liver disease.

25 Chronic depression of GH levels can also impair health.

Growth Hormone Antagonists:

In view of the foregoing, it has been suggested that if a subject is suffering from excessive GH activity, it can be useful to inhibit such activity by inhibiting the 30 production, release or action of GH, or facilitating the elimination of GH.

Among the agents useful for this purpose are those which are competitive binding antagonists of GH. It was discovered that certain mutants of the third alpha helix of 35 GH are useful for this purpose. Kopchick, USP 5,350,836.

In order to determine whether it is appropriate to

initiate or terminate use GH antagonists or other GH-inhibiting drugs, it is important to be able to monitor GH activity.

Monitoring of GH Activity:

5 The most straightforward marker of GH activity is the serum level of GH per se. For humans, the mean GH concentration (ug/L) in blood is

| | | |
|----|------------------|----------------------------|
| | preadolescent | 4.6 |
| | early adolescent | 4.8 |
| 10 | late adolescent | 13.8 |
| | adult | 1.8 |
| | ISS (10y old) | 3.5 |
| | GH deficient | 1.4 |
| | IDDM (boys) | 9.0 |
| 15 | Obese (male) | 0.66 (lower than controls) |
| | Fasting | 6.7 (higher than controls) |
| | Hyperthyroid | 1.9 (higher than controls) |

ISS = idiopathic short stature, IDDM = insulin dependent diabetes mellitus

20 See Harvey (1995), supra.

While there is definitely a correlation between high levels of GH in serum, and high levels of GH activity, it must be recognized that both the total number of GH receptors, and the distribution of those receptors among the various organs, will vary from individual to individual. Hence, in determining whether an individual is suffering from excessive GH activity, and prone to develop adverse clinical sequelae, it is helpful to identify a metabolite which is produced or released in direct or indirect response to GH and, in particular, one which is substantially liver-specific so that the specific threat to liver function can be assessed.

Another marker of GH activity is insulin-like growth factor-1 (IGF-1). IGF-1 is a 70 amino acid single chain

protein, with some structural similarity to proinsulin, which is closely regulated by GH secretion. While the majority of IGF-1 synthesis occurs in the liver, many other tissues, including bone and skeletal muscle, also release 5 IGF-1 in response to GH. IGF-1 levels have been used by clinicians to confirm suspected cases of acromegaly.

However, it would be desirable to have a marker, or combination of markers, which was more liver specific than IGF-1, for use in monitoring and predicting the effect of 10 chronic elevation of GH levels on liver function. It is known that mice transgenic for IGF-1 do not develop the same abnormalities as mice transgenic for GH, in particular, they do not develop similar liver and kidney abnormalities. See Quafe, supra, and Yang, et al., Lab. Invest., 68:62-70 15 (1993).

SUMMARY OF THE INVENTION

Applicants have identified certain genes whose expression in liver cells is elevated as a result of higher than normal GH levels. In contrast, Applicants were unable 5 to identify similarly GH-regulated genes in kidney cells.

By use of nucleic acid binding agents to bind messenger RNA transcripts produced by the transcription of any of these genes (or to bind the corresponding complementary DNAs synthesized in vitro), or by use of a protein binding agent 10 to bind a protein encoded by any of these genes, it is possible to assay the level of transcription of the gene in question, or the level of expression and secretion of the corresponding protein, and to correlate such level with the level of GH activity in the liver.

15 In addition, transgenic mammals, especially mice, rats and rabbits, which overproduce these proteins may be useful as animal models of liver pathologies.

Finally, agents which inhibit expression of these proteins (i.e., antisense nucleic acids) or the binding of 20 these proteins to their receptors (by binding either the protein or the receptor) may be useful therapeutically in inhibiting the development of liver pathologies associated with the expression of that protein.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

We have determined some of the differences in the patterns of gene expression between transgenic mice with a giant phenotype and nontransgenic mice with a normal phenotype. This is indicative of the effect of overproduction of GH on the expression of other genes. GH-mediated liver pathology is presumably the result of such expression.

Results of BLAST sequence similarity analyses identified several genes in the GH TG subtraction library suggesting that they are differentially expressed in GH TG mouse liver. These include interferon- α/β receptor (IFNR $\alpha\beta$), corticosteroid binding globulin (CBG), α -fetoprotein, cytochrome P450, fetuin (Ahsg), 3- β -hydroxysteroid (3 β HSD), paraoxonase-3 (PON-3), rab8 interacting protein and coagulation factor V. We have also identified two previously unknown genes affected by GH, cDNAs 5 and 45. This differential expression has been confirmed, in the case of IFNR $\alpha\beta$ and CBG, by using the differentially expressed cDNAs as probes. 3- β -hydrosteroid dehydrogenase is down-regulated, the others are up-regulated.

Assays for expression of these genes may be useful in the diagnosis of liver pathologies. Such diagnosis is not limited to the diagnosis of liver pathologies associated with giantism or acromegaly, or with diabetes, as other causative agents may act directly or indirectly upon the same genes.

Liver pathologies include:

1. Liver cirrhosis (hepatic disease of various etiology) such as
 - Alcoholic liver disease
 - portal hypertension
2. Liver tumor:
 - Benign: adenomas and focal nodular hyperplasia
 - Malignant: primary carcinomas and metastatic tumors

3. Infections of the liver: viral hepatitis and liver abscesses of whatever origin
4. Hepatic failure or deterioration of the liver function due to some chronic progressive disorder or acute injuries or massive necrosis
5. Drug related liver injury due to hepatotoxicity of therapeutic agents

Reference to the pathologies is: Cotran, R.S., Kumar, V.,
10 Robbins, S.L. 1989. *Robbin's Pathologic Basis of Disease* (4th ed.) W.B. Saunders Co.; Philadelphia, PA; pp. 911-980.

By preliminary screening assays using nucleic acids, antibodies, or other binding agents, carried out on mRNA, cDNA or protein samples from cells of various livers with known pathologic lesions, we may determine whether the level 15 of expression of each of the genes mentioned above is correlated with the presence (or degree of severity) of a particular liver lesion.

Also, we may make transgenic mammals (e.g. mice) that 20 overexpress the cDNA in a liver-specific manner (using a liver-specific promoter like the albumin or PEPCK promoter), and determine if these transgenic mammals develop liver histopathologies, or other signs of aging (GH transgenic mice die prematurely of liver and kidney disease).

Conversely, transgenic mammals in which expression of these genes is knocked out can be examined to determine if they provide any protection to the liver against any of the agents known to cause liver pathology, e.g., viral infection (esp. hepatitis), alcoholism, hepatotoxic drugs, tumors, etc. 25 if so, then an agent interfering with the expression or activity of the gene product would have therapeutic value.

The proteins of interest include both secreted and intracellular proteins.

Secreted proteins can potentially disrupt normal 35 signaling mechanisms through ligand/receptor interaction. They can also be used as indicators of a pathophysiological state. Also, they may be "peptide hormones". Thus, they could have diagnostic or therapeutic value. Depending upon

the scenario, recombinant agonists or antagonists may emerge from these molecules.

Intracellular proteins, on the other hand, could regulate the intrinsic biological functions of certain cells. These proteins could be potential drug targets in that one may design molecules to activate or inhibit them.

1. α -fetoprotein- Closely related to serum albumin but is found primarily during fetal development, during which elevated levels can be indicative of neural tube defects. Elevated levels have been reported in patients with alcoholic liver disease and hepatocellular carcinoma (HCC). [Scand J Gastroenterol 2000 Mar;35(3):333-6] This is a secreted protein.

15 2. Fetuin (AHSG)- A 52 kDa glycoprotein that has been reported to be an inhibitor of the insulin receptor tyrosine kinase. [Kalabay L, Horm Metab Res 1998 Jan;30(1):1-6] AHSG has also been reported to inhibit protease activities and to act as a regulator 20 of calcium metabolism and osteogenesis. [Banine F, et al. Eur J Biochem 2000 Feb;267(4):1214-22] This protein may be important in GH's diabetogenic activity. Elimination or down regulation of this activity may allow cells to become more sensitive to the action of 25 insulin. Thus, inhibitors of this action could be used as "insulin sensitizers".

30 3. 3- β -Hydroxysteroid Dehydrogenase (3- β -HSD)- Isomerase and Dehydrogenase that plays an important role in all aspects of steroid production. It is present in many different isoforms which indicates multiple functionality. It acts in the liver as a key enzyme in the cholesterol biosynthetic pathway and as a transporter of bile acids [Marscall HU, et al. Hepatology 2000 Apr;31(4):990-6] It has also been 35 reported that GH administration to cultured cells stimulated the activity of 3- β -HSD. [Gregoraszczuk EL, et al. Anim Reprod Sci 2000 Feb 28;58(1-2):113-25]

Since the activity goes up in the livers of these GH animals and since it has been shown to be involved in cholesterol synthesis, it could be used as a target for the down regulation of cholesterol production.

5

4. Rab8 interacting protein- Rab proteins are small GTP binding proteins involved in vesicular transport during endocytosis and exocytosis. They are distant relatives of the *ras* family of oncogenes, but are not oncogenic themselves. Rab8ip shows similarity to the GC kinase, a serine/threonine kinase that has recently been identified in stress activated human lymphoid tissue. It is thought that Rab8ip may have a role in modulation of secretion in response to stress stimuli.

10

15

[Ren M, et al. *Proc Natl Acad Sci USA* 1996 May 14;93(10):5151-5]

20

25

30

5. Paraoxonase 3 (PON3)- Although little is known about PON3, the PON family of gene products are active in cholesterol biosynthesis. PON1 is an enzyme found in serum which is associated with high density lipoprotein (HDL) and is thought to protect low density lipoprotein (LDL) from peroxidation. Decreased activity of PON enzymes is found in sufferers of chronic renal failure. [Dantoine TF, *J Am Soc Nephrol* 1998 Nov;9(11):2082-8] There has been recent speculation as to the merit of potential testing for genetic variation in the PON gene family or whether the gene products might be good candidates for therapeutic interventions. [Hegele RA, *Ann Med* 1999 Jun;31(3):217-24]

6. S-2-hydroxy acid oxidase (Glycolate oxidase)-

This gene was just recently cloned in mice. [Kohler SA, *J Biol Chem* 1999 Jan 22;274(4):2401-7] It is a peroxisomal protein that is involved in the oxidation of hydroxy acids such as L-lactate. Any method to reduce lactic acid in a diabetic individual would be

beneficial.

7. Interferon α/β receptor (IFN $\alpha\beta$ R)- Interferons are antiviral, antiproliferative, immune responsive cytokines. Recombinant forms have been in use for the treatment of various malignancies. Serum levels of soluble IFN $\alpha\beta$ R have been found to be elevated in patients with chronic hepatitis C. [Mizukoshi E, et al. *Hepatology* 1999 Nov;30(5):1325-31] It is thought that resistance to IFN therapy in patients with chronic hepatitis C may be due to low levels of hepatic IFN $\alpha\beta$ R. [Yatsuhashi H, *J Hepatol* 1999 Jun;30(6):995-1003]. Thus any method by which this IFN "binding protein" would be increased could be beneficial. Since the soluble version of this has been found, and it is secreted, it could be used as a diagnostic marker.
- 10
8. Growth Hormone Receptor (GHR)- All physiological attributes of growth hormone are mediated via signaling through binding with the GHR. Low levels of GHR have been indicated in cirrhotic liver. [Shen XY, *J Clin Endocrinol Metab* 1998 Jul;83(7):2532-8]
- 20
9. Cytochrome P450- The cytochromes are an extensive family of Heme containing electron transport molecules found in liver microsomes. They convert a wide range of substrates to forms that are more easily excreted by the cell, some of which may be carcinogenic. The cytochromes are also involved in steroid and prostaglandin biosynthesis.
- 25
10. Proteosome subunit Z- A component of the multicatalytic Proteinase complex found in the eukaryotic cytosol and nucleus that is responsible for ubiquitin dependent protein degradation. It has recently been reported that GHR internalization requires proteosome action and active ubiquitin conjugation system. [van Kerkhof P, *J Biol Chem* 2000
- 30

11

Jan 21;275(3):1575-80]. Any substance that could control ubiquitination could be of value.

11. Corticosteroid Binding Globulin (CBG)- The major function of CBG is to regulate the bioavailability of plasma cortisol by restriction it's exit from the capillaries. [Alexander SL, *J Endocrinol* 1998 Jun;157(3):425-32] CBG is regulated by many factors, including stress, steroid sex hormones, and GH (when dosed continuously). [Jansson JO, *J Endocrinol* 1989 Sep;122(3):725-32]

12. Coagulation Factor V- Coagulation factors are a group of protease enzymes and cofactors involved in clotting. Their activation is triggered by tissue injury and phospholipoprotein release, which ultimately leads to the production of thrombin. Again, any substance that could up or down regulated blood clotting could be of value.

Definitions

Two proteins are cognate if they are produced in different species, but are sufficiently similar in structure and biological activity to be considered the equivalent proteins for those species. If the accepted scientific names for two proteins are the same but for the species identification (e.g., human GH and shark GH), they should be considered cognate. If not, the two proteins may still be considered cognate if they have at least 50% amino acid sequence identity (when globally aligned with a pam250 scoring matrix with a gap penalty of the form $q+r(k-1)$ where k is the length of the gap, $q=-12$ and $r=-4$; percent identity=number of identities as percentage of length of shorter sequence) and at least one biological activity in common.

Two genes are cognate if they are expressed in different species and encode cognate proteins.

35 Gene expression may be said to be specific to a

particular tissue if the average ratio of the specific mRNA to total mRNA for the cells of that tissue is at least 10% higher than the average ratio is for the cells of some second tissue. Absolute specificity is not required.

- 5 Hence, a gene may be said to be expressed specifically in more than one tissue.

When the term "specific" is used in this specification, absolute specificity is not intended, merely a detectable difference.

- 10 Preferably the markers of the present invention are, singly or in combination, more specific to the target tissue than are serum GH or IGF-1 levels, or than GH mRNA or IGF-1 mRNA levels in the target tissue.

- If this specification calls for alignment of DNA sequences, and one of the sequences is intended for the use as a hybridization probe, the sequences are to be aligned using a local alignment program with matches scored +5, mismatches scored -4, the first null of a gap scored -12, and each additional null of the same gap scored -2.
- 20 Percentage identity is the number of identities expressed as a percentage of the length of the overlap, including internal gaps.

In Vitro Assays

- The in vitro assays of the present invention may be applied to any suitable analyte-containing sample, and may be qualitative or quantitative in nature.

- For the techniques to practice these assays, see, in general, Ausubel, et al., Current Protocols in Molecular Biology, and in particular chapters 2 ("Preparation and Analysis of DNA"), 3 ("Enzymatic Manipulation of DNA and RNA"), 4 ("Preparation and Analysis of RNA"), 5 ("Construction of Recombinant DNA libraries") 6 ("Screening of Recombinant DNA Libraries"), 7 ("DNA Sequencing"), 10 ("Analysis of Proteins"), 11 ("Immunology"), 14 ("In situ hybridization and immune histochemistry"), 15 ("The Polymerase Chain Reaction"), 19 ("Informatics for Molecular Biologists"), and 20 ("Analysis of Protein Interactions").

Also see, in general, Coligan, et al., Current Protocols in Immunology, and in particular, chapters 2 ("Induction of immune responses"), 8 ("Isolation and Analysis of Proteins"), 9 ("Peptides"), 10 ("Molecular Biology") and 17 5 ("Engineering Immune Molecules and Receptors"). Also see Coligan, et al., Current Protocols in Protein Science.

The Assay Target (Marker) (Analyte)

In one embodiment, the assay target is a messenger RNA transcribed from a gene which, in liver cells, has increased 10 transcriptional activity if serum GH levels are increased. This messenger RNA may be a full length transcript of the gene, or merely a partial transcript. In the latter case, it must be sufficiently long so that it is possible to achieve specific binding, e.g., by nucleic acid 15 hybridization. For the purpose of conducting the assay, the messenger RNA is extracted from liver cells by conventional means. Alternatively, the assay target may be a complementary DNA synthesized in vitro from the messenger RNA as previously described.

20 For convenience, the term "gene" or "target sequence" will be used to refer to the messenger RNA or complementary DNA corresponding to the induced gene, and to the coding gene proper.

In another embodiment, the assay target is a protein 25 encoded by said gene and expressed at higher levels in response to elevated GH levels. If the protein is secreted, the assay may be performed on serum. If the protein is not secreted, then liver cells will be obtained from the subject and lysed to expose the cytoplasmic contents.

30 In either embodiment, one or more purification steps may be employed prior to the practice of the assay in order to enrich the sample for the assay target.

The proteins of particular interest are as follows:
alpha-fetoprotein
35 fetuin
3- β -hydroxysteroid
rab8-interacting protein

paraoxonase-3
interferon α/β receptor
proteasome z-subunit
corticosteroid binding globulin
growth hormone receptor
cytochrome P450IIIA
cytochrome P450
coagulation factor V
S-2 hydroxyacid oxidase

The genes of particular interest are those encoding the above proteins. These genes were identified, as described in Example 1, on the basis of the identity or similarity of mouse cDNAs obtained by subtractive hybridization methods to known mouse genes or cDNAs (or, in the case of the S-2 hydroxyacid oxidase, to a known rat gene). The mouse sequences were transferred onto a nylon membrane. After transfer of RNA onto the membrane, the membrane may then be used in a hybridization reaction with a suitable probe, which may be a synthetic probe directed against a gene already known to be a marker, or which may be a cDNA probe prepared directly from subtractive hybridization, wherein the fragment encoding the gene of interest, that is enriched in GH-overproducing subjects, will be labeled, preferably either radioactively with ^{32}P or non-radioactively with DIG (Digoxigenin). A negative control, such as one composed of RNA sample from liver of normal subjects, may be resolved side by side with the patients' sample. Detection of this gene or protein could therefore indicate the presence of liver problem.

Certainly newly discovered DNAs are also of interest. These are identified below as clones 5 and 45. The proteins encoded by the ORFs embedded in these DNAs are also of interest.

Samples

The sample may be of any biological fluid or tissue which is reasonably expected to contain the messenger RNA transcribed from one of the above genes, or a protein

expressed from one of the above genes. The sample may be of liver tissue or interstitial fluid, or of a systemic fluid into which liver proteins are secreted.

A non-invasive sample collection will involve the use
5 of urine samples from human subjects. Blood samples will also be obtained in order to obtain plasma or serum from which secreted proteins can be evaluated. Liver aspirates can also be obtained to detect for the presence of genes and proteins of interest. The most invasive method would
10 involve obtaining liver biopsies.

Analyte Binding Reagents (Molecules, ABM)

When the assay target is a nucleic acid, the preferred binding reagent is a complementary nucleic acid. However, the nucleic acid binding agent may also be a peptide or
15 protein. A peptide phage library may be screened for peptides which bind the nucleic acid assay target. In a similar manner, a DNA binding protein may be randomly mutagenized in the region of its DNA recognition site, and the mutants screened for the ability to specifically bind
20 the target. Or the hypervariable regions of antibodies may be mutagenized and the antibody mutants displayed on phage.

When the assay target is a protein, the preferred binding reagent is an antibody, or a specifically binding fragment of an antibody. The antibody may be monoclonal or
25 polyclonal. It can be obtained by first immunizing a mammal with the protein target, and recovering either polyclonal antiserum, or immunocytes for later fusion to obtain hybridomas, or by constructing an antibody phage library and screening the antibodies for binding to the target. The
30 binding reagent may also be a binding molecule other than an antibody, such as a receptor fragment, an oligopeptide, or a nucleic acid. A suitable oligopeptide or nucleic acid may be identified by screening a suitable random library.

Binding and Reaction Assays

35 The assay may be a binding assay, in which one step involves the binding of a diagnostic reagent to the analyte,

or a reaction assay, which involves the reaction of a reagent with the analyte. The reagents used in a binding assay may be classified as to the nature of their interaction with analyte: (1) analyte analogues, or (2) analyte binding molecules (ABM). They may be labeled or insolubilized.

In a reaction assay, the assay may look for a direct reaction between the analyte and a reagent which is reactive with the analyte, or if the analyte is an enzyme or enzyme inhibitor, for a reaction catalyzed or inhibited by the analyte. The reagent may be a reactant, a catalyst, or an inhibitor for the reaction.

An assay may involve a cascade of steps in which the product of one step acts as the target for the next step. These steps may be binding steps, reaction steps, or a combination thereof.

Signal Producing System (SPS)

In order to detect the presence, or measure the amount, of an analyte, the assay must provide for a signal producing system (SPS) in which there is a detectable difference in the signal produced, depending on whether the analyte is present or absent (or, in a quantitative assay, on the amount of the analyte). The detectable signal may be one which is visually detectable, or one detectable only with instruments. Possible signals include production of colored or luminescent products, alteration of the characteristics (including amplitude or polarization) of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a component or product. The term "signal" is intended to include the discontinuance of an existing signal, or a change in the rate of change of an observable parameter, rather than a change in its absolute value. The signal may be monitored manually or automatically.

In a reaction assay, the signal is often a product of the reaction. In a binding assay, it is normally provided by a label borne by a labeled reagent.

Labels

The component of the signal producing system which is most intimately associated with the diagnostic reagent is called the "label". A label may be, e.g., a radioisotope, a 5 fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, an agglutinable particle.

The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful 10 for the purpose of the present invention are ^3H , ^{32}P , ^{125}I , ^{131}I , ^{35}S , ^{14}C , and, preferably, ^{125}I .

The label may also be a fluorophore. When the fluorescently labeled reagent is exposed to light of the proper wave length, its presence can then be detected due to 15 fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, α -phthaldehyde and fluorescamine.

Alternatively, fluorescence-emitting metals such as 20 ^{125}Eu , or others of the lanthanide series, may be incorporated into a diagnostic reagent using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) of ethylenediamine-tetraacetic acid (EDTA).

The label may also be a chemiluminescent compound. The 25 presence of the chemiluminescently labeled reagent is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isolumino, theromatic acridinium ester, 30 imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used for labeling. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. 35 The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Enzyme labels, such as horseradish peroxidase and alkaline phosphatase, are preferred. When an enzyme label is used, the signal producing system must also include a substrate for the enzyme. If the enzymatic reaction product 5 is not itself detectable, the SPS will include one or more additional reactants so that a detectable product appears.

An enzyme analyte may act as its own label if an enzyme inhibitor is used as a diagnostic reagent.

Conjugation Methods

10 A label may be conjugated, directly or indirectly (e.g., through a labeled anti-ABM antibody), covalently (e.g., with SPDP) or noncovalently, to the ABM, to produce a diagnostic reagent. Similarly, the ABM may be conjugated to a solid phase support to form a solid phase 15 ("capture") diagnostic reagent.

Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either 20 soluble to some extent or insoluble for the purposes of the present invention.

The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to its target. Thus the support 25 configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

Binding Assay Formats

30 Binding assays may be divided into two basic types, heterogeneous and homogeneous. In heterogeneous assays, the interaction between the affinity molecule and the analyte does not affect the label, hence, to determine the amount or presence of analyte, bound label must be separated from free 35 label. In homogeneous assays, the interaction does affect the activity of the label, and therefore analyte levels can

be deduced without the need for a separation step.

In one embodiment, the ABM is insolubilized by coupling it to a macromolecular support, and analyte in the sample is allowed to compete with a known quantity of a labeled or 5 specifically labelable analyte analogue. The "analyte analogue" is a molecule capable of competing with analyte for binding to the ABM, and the term is intended to include analyte itself. It may be labeled already, or it may be labeled subsequently by specifically binding the label to a 10 moiety differentiating the analyte analogue from analyte. The solid and liquid phases are separated, and the labeled analyte analogue in one phase is quantified. The higher the level of analyte analogue in the solid phase, i.e., sticking to the ABM, the lower the level of analyte in the 15 sample.

In a "sandwich assay", both an insolubilized ABM, and a labeled ABM are employed. The analyte is captured by the insolubilized ABM and is tagged by the labeled ABM, forming a ternary complex. The reagents may be added to the sample 20 in either order, or simultaneously. The ABMs may be the same or different. The amount of labeled ABM in the ternary complex is directly proportional to the amount of analyte in the sample.

The two embodiments described above are both 25 heterogeneous assays. However, homogeneous assays are conceivable. The key is that the label be affected by whether or not the complex is formed.

Detection of Genes of Interest

For the detection of genes in the sample, PCR can be 30 performed using primers specific for the genes of interest. This would amplify the genes of interest. Primers may be designed to anneal to any site within the open reading frames of the genes of interest. Resolution of the fragments by electrophoresis on agarose gel may be used to determine 35 the presence of the genes. PCR product may be quantitated by densitometry in order to estimate the concentration of the genes in the samples.

Detection of genes of interest may also be done by **Northern blot analysis** on liver biopsies. Tissue sample from patients may be obtained and the total RNA extracted using RNAStat 60. The total RNA sample may then be resolved 5 on denaturing gel by electrophoresis and then transferred onto a nylon membrane. After transfer of RNA onto the membrane, the membrane may then be used in hybridization with a suitable probe, which may be a synthetic probe directed against a gene already known to be a marker, or 10 which may be a cDNA probe prepared directly from subtractive hybridization, wherein the fragment encoding the gene of interest, that is enriched in GH-overproducing subjects, will be labeled, preferably either radioactively with ^{32}P or non-radiactively with DIG (Digoxigenin). A 15 negative control, such as one composed of RNA sample from liver of normal subjects, may be resolved side by side with the patients' sample, to determine quantitatively whether there is a significant increase in the level of gene expression. Elevation of the messenger RNA transcript from 20 this gene would imply that liver damage might have occurred.

The DNA sequences of the present invention may be used either as hybridization probes per se, or as primers for PCR.

In a hybridization assay, a nucleic acid reagent may be 25 used either as a probe, or as a primer. For probe use, only one reagent is needed, and it may hybridize to all or just a part of the target nucleic acid. Optionally, more than one probe may be used to increase specificity. For the primer-based assay, two primers are needed. These hybridize the 30 non-overlapping, separated segments of the target sequence. One primer hybridizes to the plus strand, and the other to the minus strand. By PCR techniques, the target nucleic acid region starting at one primer binding site and ending at the other primer binding site, along both strands, is 35 amplified, including the intervening segment to which the primers do not hybridize. In a primer-based assay, the primer thus will not correspond to the entire target, but rather each primer will correspond to one end of the target

sequence.

In probe-based assays, hybridizations may be carried out on filters or in solutions. Typical filters are nitrocellulose, nylon, and chemically-activated papers. The 5 probe may be double stranded or single stranded, however, the double stranded nucleic acid will be denatured for binding.

To be successful, a hybridization assay, whether primer- or probe-based, must be sufficiently sensitive and 10 specific to be diagnostically useful.

For probe-based assays, sensitivity is affected by the amount and specific activity of the probe, the amount of the target nucleic acid, the detectability of the label, the rate of hybridization, and the duration of the 15 hybridization. The hybridization rate is maximized at a Ti (incubation temperature) of 20-25°C. below Tm for DNA:DNA hybrids and 10-15°C. below Tm for DNA:RNA hybrids. It is also maximized by an ionic strength of about 1.5M Na⁺. The rate is directly proportional to duplex length and inversely 20 proportional to the degree of mismatching.

For primer-based PCR assays, sensitivity is not usually a major issue because of the extreme amplification of the signal.

For probe-based assays, specificity is a function of 25 the difference in stability between the desired hybrid and "background" hybrids. Hybrid stability is a function of duplex length, base composition, ionic strength, mismatching, and destabilizing agents (if any).

The Tm of a perfect hybrid may be estimated.
30 for DNA:DNA hybrids, as

$$T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$$

and for DNA:RNA hybrids, as

$$T_m = 79.8^\circ\text{C} + 18.5 (\log M) + 0.58 (\%GC) - 11.8 (\%GC)^2 - 0.56(\% \text{ form}) - 820/L$$

where

M, molarity of monovalent cations, 0.01-0.4 M NaCl,

%GC, percentage of G and C nucleotides in DNA, 30%-75%,
and
% form, percentage formamide in hybridization solution,

L, length hybrid in base pairs.

5 Tm is reduced by 0.5-1.5°C for each 1% mismatching.

Tm may also be estimated by the method of Tinoco et al., developed originally for the determination of the stability of a proposed secondary structure of an RNA. Tm may also be determined experimentally.

10 Filter hybridization is typically carried out at 68°C., and at high ionic strength (e.g., 5 - 6 x SSC), which is nonstringent, and followed by one or more washes of increasing stringency, the last was being of the ultimately desired stringency. The equations for Tm can be used to
15 estimate the appropriate Ti for the final wash, or the Tm of the perfect duplex can be determined experimentally and Ti then adjusted accordingly.

While a mouse cDNA was used to probe a mouse liver cDNA library, and could be used to probe nonmurine liver cDNA
20 libraries, it would be expected that there would be some sequence divergence between cognate mouse and nonmouse DNAs, possibly as much as 25-50%.

Hence, when the human DNA cognate to the original mouse cDNA is known, it is better to use that DNA, or a fragment
25 thereof, to probe a human liver cDNA library. The practitioner may use the complete genomic DNA or cDNA sequence of the human gene as a probe, or, for the sake of greater specificity or synthetic convenience, a partial sequence.

30 It is also noted that while some of the mouse clones were identical to subsequences of a databank mouse DNA, others diverged slightly. This divergence (up to 5%) could be artifactual (sequencing error) or real (allelic variation).

35 Hybridization conditions should be chosen so as to permit allelic variations, but avoid hybridizing to other genes. In general, stringent conditions are considered to

be a T_m of 5°C. below the T_m of a perfect duplex, and a 1% divergence corresponds to a 1-1.5°C. reduction in T_m . Hence, use of a T_m of 5-15°C. below the T_m of the double stranded form of the probe is recommended.

- 5 If the sequences of the major allelic variants are known, one may use a mixed probe, and optionally increase the stringency.

If there is no known human gene cognate to the mouse (or rat) gene homologous to the clone, then the mouse (or 10 rat) gene, or other known nonhuman cognate gene, may be used as a probe. In this case, more moderate stringency hybridization conditions should be used. The nonhuman gene may be modified to obey a more human set of codon preferences.

- 15 Alternatively, the mouse (or rat) gene may be used once as a probe to isolate the human gene, and the human gene then used for diagnostic work. If a partial human cDNA is obtained, it may be used to isolate a larger human cDNA, and the process repeated as needed until the complete human cDNA 20 is obtained.

For cross-species hybridization, the T_i should be reduced further, by about 0.5-1.5°C., e.g., 1°C, for each expected 1% divergence in sequence. The degree of divergence may be estimated from the known divergence of the 25 most closely related pairs of known genes from the two species.

- If the desired degree of mismatching results in a wash temperature less than 45°C., it is desirable to increase the salt concentration so a higher temperature can be used.
30 Doubling the SSC concentration results in about a 17°C. increase in T_m , so washes at 45°C in 0.1 x SSC and 62°C in 0.2 x SSC are equivalent (1 x SSC = 0.15 M NaCl, 0.015M trisodium citrate, pH 7.0).

The person skilled in the art can readily determine 35 suitable combinations of temperature and salt concentration to achieve this degree of stringency.

The hybridization conditions set forth in the Examples may be used as a starting point, and then made more or less

stringent as the situation merits.

Examples of successful cross-species-hybridization experiments include Braun, et al., EMBO J., 8:701-9 (1989) (mouse v. human), Imamura, et al., Biochemistry, 30:5406-11 (1991) (human v. rat), Oro, et al., Nature, 336:493-6 (1988) (human v. Drosophila), Higuti, et al., Biochem. Biophys. Res. Comm., 178:1014-20 (1991) (rat v. human), Jeung, et al., FEBS Lett., 307:224-8 (1992) (rat, bovine v. human), Iwata, et al., Biochem. Biophys. Res. Comm., 182:348-54 (1992) (human v. mouse), Libert, et al., Biochem. Biophys. Res. Comm., 187:919-926 (1992) (dog v. human), Wang, et al., Mamm. Genome, 4:382-7 (1993) (human v. mouse), Jakubiczka, et al., Genomics, 17:732-5 (1993) (human v. bovine), Nahmias, et al., EMBO J., 10:3721-7 (1991) (human v. mouse), Potier, et al., J. DNA Sequencing and Mapping, 2:211-218 (1992) (rat v. human), Chan, et al., Somatic Cell Molec. Genet., 15:555-62 (1989) (human v. mouse), Hsieh, et al., Id., 579-590 (1989) (human, mouse v. bovine), Sumimoto, et al., Biochem. Biophys. Res. Comm., 165:902-6 (1989) (human v. mouse), Boutin, et al., Molec. Endocrinol., 3:1455-61 (1989) (rat v. human), He, et al., Biochem. Biophys. Res. Comm., 171:697-704 (1990) (human, rat v. dog, guinea pig, frog, mouse), Galizzi, et al., Int. Immunol., 2:669-675 (1990) (mouse v. human). See also Gould, et al., Proc. Natl. Acad. Sci. USA, 86:1934-8 (1989).

In general, for cross-species hybridization, $T_i = 25 - 35^\circ\text{C}$. below T_m . Wash temperatures and ionic strengths may be adjusted empirically until background is low enough.

For primer-based PCR assays, the specificity is most dependent on reagent purity.

The final considerations are the length and binding site of the probe. In general, for probe-based assays, the probe is preferably at least 15, more preferably at least 20, still more preferably at least 50, and most preferably at least 100 bases (or base pairs) long. Preferably, if the probe is not complementary to the entire gene, it targets a region low in allelic variation.

In general, for primer-based PCR assays, the primer is

preferably at least 18-30 bases in length. Longer primers do no harm, shorter primers may sacrifice specificity. The distance between the primers may be as long as 10 kb, but is preferably less than 3kb, and of course should taken into account the length of the target sequence (which is likely to be shorter for mRNA or cDNA than for genomic DNA). Preferably, primers have similar GC content, minimal secondary structure, and low complementarily to each other, particularly in the 3' region.

10 For theoretical analysis of probe design considerations, see Lathe, et al., J. Mol. Biol., 183:1-12 (1985).

Detection of Proteins of Interest

15 ELISA can be done on blood plasma or serum from patients using antibodies specific to the protein of interest. Samples will be incubated with primary antibodies on plates. This primary antibody is specific to the protein of interest.

Another method that can be conducted will involve the
20 use of chemical or enzymatic reactions in which the protein of interest will act as a substrate (or, if the protein is an enzyme, as a catalyst) to cause a reaction that lead to the production of colored solution or emission of fluorescence. **Spectrometric** analysis can be done in order to
25 determine the concentration of the proteins in the sample.

Western blot analysis can also be done on the plasma/serum, liver aspirate, liver biopsies or urine samples. This would involve resolving the proteins on an electrophoretic gel, such as an SDS PAGE gel, and
30 transferring the resolved proteins onto a nitrocellulose or other suitable membrane. The proteins are incubated with a target binding molecule, such as an antibody.

This binding reagent may be labeled or not. If it is unlabeled, then one would also employ a secondary, labeled
35 molecule which binds to the binding reagent. One approach involves avidinating one molecule and biotinylating the other. Another is for the secondary molecule to be a

secondary antibody which binds the original binding reagent.

To improve detection of the specific protein, **immunoprecipitation** can be conducted. This typically will involve addition of a monoclonal antibody against the 5 protein of interest to samples, then allowing the Ig-protein complex to precipitate after the addition of an affinity bead (ie antihuman Ig sepharose bead). The immunoprecipitates will undergo several washings prior to transfer onto a nitrocellulose membrane. The Western blot 10 analysis can be perform using another antibody against the primary antibody used.

Interpretation of Assay Results

The assay may be used to predict the clinical state of 15 the liver if the level of GH activity remains unchanged.

A scheme for the diagnostic interpretation of the level of the target in question is determined in a conventional manner by monitoring the level of GH, the level 20 of the target, and the liver condition in a suitable number of patients, and correlating the level of the target at an earlier time point with the simultaneous or subsequent liver tissue state.

This correlation is then used to predict the future clinical state of the liver in new patients with high GH 25 levels.

The diagnosis may be based on a single marker, or upon a combination of markers, which may include, besides the markers mentioned above, the level of GH or of IGF-1. A suitable combination may be identified by any suitable 30 technique, such as multiple regression, factor analysis, or a neural network using the scaled levels of the markers as inputs and the current or subsequent liver state as an output.

In vivo Diagnostic Uses

35 Radio-labelled ABM which are not rapidly degraded in blood may be administered to the human or animal subject. Administration is typically by injection, e.g., intravenous

or arterial or other means of administration in a quantity sufficient to permit subsequent dynamic and/or static imaging using suitable radio-detecting devices. The dosage is the smallest amount capable of providing a diagnostically effective image, and may be determined by means conventional in the art, using known radio-imaging agents as a guide.

Typically, the imaging is carried out on the whole body of the subject, or on that portion of the body or organ relevant to the condition or disease under study. The amount of radio-labelled ABM accumulated at a given point in time in relevant target organs can then be quantified.

A particularly suitable radio-detecting device is a scintillation camera, such as a gamma camera. A scintillation camera is a stationary device that can be used to image distribution of radio-labelled ABM. The detection device in the camera senses the radioactive decay, the distribution of which can be recorded. Data produced by the imaging system can be digitized. The digitized information can be analyzed over time discontinuously or continuously. The digitized data can be processed to produce images, called frames, of the pattern of uptake of the radio-labelled ABM in the target organ at a discrete point in time. In most continuous (dynamic) studies, quantitative data is obtained by observing changes in distributions of radioactive decay in target organs over time. In other words, a time-activity analysis of the data will illustrate uptake through clearance of the radio-labelled binding protein by the target organs with time.

Various factors should be taken into consideration in selecting an appropriate radioisotope. The radioisotope must be selected with a view to obtaining good quality resolution upon imaging, should be safe for diagnostic use in humans and animals, and should preferably have a short physical half-life so as to decrease the amount of radiation received by the body. The radioisotope used should preferably be pharmacologically inert, and, in the quantities administered, should not have any substantial physiological effect.

The ABM may be radio-labelled with different isotopes of iodine, for example ^{123}I , ^{125}I , or ^{131}I (see for example, U.S. Patent 4,609,725). The extent of radio-labeling must, however be monitored, since it will affect the calculations made based on the imaging results (i.e. a diiodinated ABM will result in twice the radiation count of a similar monoiodinated ABM over the same time frame).

In applications to human subjects, it may be desirable to use radioisotopes other than ^{125}I for labelling in order to decrease the total dosimetry exposure of the human body and to optimize the detectability of the labelled molecule (though this radioisotope can be used if circumstances require). Ready availability for clinical use is also a factor. Accordingly, for human applications, preferred radio-labels are for example, $^{99\text{m}}\text{Tc}$, ^{67}Ga , ^{68}Ga , ^{90}Y , ^{111}In , $^{113\text{m}}\text{In}$, ^{123}I , ^{186}Re , ^{188}Re or ^{211}At .

The radio-labelled ABM may be prepared by various methods. These include radio-halogenation by the chloramine - T method or the lactoperoxidase method and subsequent purification by HPLC (high pressure liquid chromatography), for example as described by J. Gutkowska et al in "Endocrinology and Metabolism Clinics of America: (1987) 16 (1):183. Other known method of radio-labelling can be used, such as IODOBEADS™.

There are a number of different methods of delivering the radio-labelled ABM to the end-user. It may be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. Because proteins are subject to being digested when administered orally, parenteral administration, i.e., intravenous subcutaneous, intramuscular, would ordinarily be used to optimize absorption of an ABM, such as an antibody, which is a protein.

EXAMPLES

BLASTIN and BLASTP searches were performed with the default parameters match +1, mismatch -3, gap q=-5 r=-2, penalty q+rk for gap length k. For BLASTP, BLOSUM62 matrix 5 with q=-1, r=-1, lambda ratio=0.85.

Preliminary results indicate that proteosome z-subunit, GH receptor, rab8 interacting protein, alpha-fetoprotein, fetuin are elevated in livers of GH TM when compared to NT, whereas, 3-beta-HSD is decreased. IFNR, CBG, clone 45 and 10 clone 5 are expressed in GH TM and not in NT littermates.

Example 1Introduction: GH-inducible Liver Genes for Diagnosis of GH Action on Liver Pathology

Human growth hormone (hGH) upon binding to its receptor 15 induces expression of a number of genes. These growth hormone (GH)-inducible genes can be identified in transgenic mice (TM) expressing bovine GH (bGH). These mice are twice as big as wild type (WT) mice and are also reported to show some form of liver pathology in their later stages of life. 20 Our work aimed to create a library of liver GH inducible-genes in liver and to identify genes that are associated with the progression of liver disease that may eventually be used to diagnose pathologic liver in humans as observed on patients with acromegaly, liver cirrhosis, and viral 25 infections causing hepatitis.

Production of differentially expressed cDNAs from GH TM by Subtractive Hybridization

The method employed to determine the GH-inducible genes in bGH TM involves subtractive hybridization using 30 Clontech's PCR-Select cDNA Subtraction kit. This method requires that mRNAs be isolated first and then converted into cDNAs. The mRNAs from liver of 60 days old bGH TM and WT mice were isolated by passing through oligo-dT columns (Invitrogen's Fastract 2.0) total RNAs prepared by RNAStat 35 60. Conversion of mRNAs to cDNAs involves the use of AMV reverse transcriptase (Clontech). The primer used for the

CTAATACGACTCACTATAAGGC-3', bases 1-22 of SEQ ID NO:1) were generated.

Subtraction is achieved by preventing the tester-driver hybrid sequences from being amplified during PCR

5 amplification while hybrids between testers with adaptor 1 and adaptor 2R can. Thus, those cDNA fragments that undergo PCR amplification correspond to differentially expressed GH TM.

Two steps of PCR amplification were conducted to enrich
10 the pool of differentially expressed cDNA from GH TM. PCR primer 1 was used in the first PCR amplification at 94°C for 25 sec followed by 27 cycles at three different temperatures of 94°C for 10 sec, 66°C for 30 sec, and 72°C for 1.5 min.

After the first PCR amplification step which resulted to
15 exponential amplification of differentially expressed sequences from GH TM, nested PCR primer 1(5'-TCGAGCGGCCGCCGGCAGGT-3', bases 23-44 of SEQ ID NO:1) and nested PCR primer 2R(5'-AGCGTGGTCGCGGCCGAGGT-3', bases 23-42 of SEQ ID NO:3) were added to the first PCR amplified
20 reaction mixture. Then the second PCR amplification step was conducted at 10-12 cycles of amplification at 94°C for 10 sec, 68°C for 30 sec and 72°C for 1.5 min. to further enrich the differentially expressed sequence from GH TM. The integrity of the products from each manipulation was
25 determined by gel electrophoresis of an aliquot of the reaction mixtures. The differentially expressed sequences obtained by subtractive hybridization were subcloned directly into PCR II cloning vector.

Subcloning and Sequencing of Differentially Expressed

30 Subtraction Products

The pool of partial cDNA fragments was ligated into a pCR® 2.1 expression vector using the TA Cloning® Kit from Invitrogen®. The ligation mixture was subsequently transformed into Library Efficiency DH5α™ Competent Cells from Life Technologies. Ampicillin resistant colonies were propagated and plasmid DNA was extracted and purified using an alkaline lysis miniprep protocol (Birnboim, H.C. 1983).

The purified plasmid DNAs containing different partial cDNA fragments were then sequenced using S labeled dNTPs and the T7 Sequenase™ version 2.0 DNA polymerase from Amersham Life Science Products. The sequencing primer, 5'

5 TACTCAAGCTATGCATCAAG 3' (SEQ ID NO:5), hybridized to the pCR® 2.1 expression vector in the multiple cloning site ~60 bases 5' of the partial cDNA insert. The sequence data was analyzed and matched against known sequences using BLAST (Basic Local Alignment Search Tools), available through the

10 National Centers for Biotechnology Information (NCBI) internet database. Our search results indicated that out of 56 sequences analyzed, 13 were identifiable as perfectly or almost perfectly identical to subsequences of known genes on the database. These GH-inducible genes in the liver of GH

15 TM are mouse α-fetoprotein, fetuin, 3-β-Hydroxysteroid, rab8-interacting protein, paraoxonase-3, interferon α/β receptor (IFNR αβ), proteasome z-subunit, corticosteroid binding globulin (CBG), growth hormone receptor, cytochrome P450IIIA, cytochrome P450, and coagulation factor V, and rat

20 S-2-hydroxyacid oxidase. It follows that the cognate human genes may be used as probes for observing GH-regulated expression of those genes in the liver, which genes are presumed to be regulated in a similar manner.

Northern Analysis of RNA Extracted from Wildtype and bGH Transgenic Mice

Total RNA was extracted and purified from the livers of both bGH transgenic and nontransgenic littermates. 60 day old mice were euthanized and dissected to obtain the tissues we needed. Tissues were then homogenized in 1ml RNA STAT-30 60™ Total RNA/mRNA Isolation Reagent per 100mg of tissue. The RNA was quantitated by spectrophotometry (O.D. 260/280) and electrophoresed on agarose-formaldehyde gels and transferred onto Boehringer-Mannheim nylon membranes. probes were generated using an EcoRI which cleaves out the 35 partial cDNA insert from the plasmid DNAs. The fragments were purified using the Qiaex® II Agarose Gel Extraction Kit from Qiagen®. The purified fragments were then labeled

using the Random Primed DNA Labeling Kit from Boehringer Mannheim. The membrane bound RNA was then hybridized with $\alpha^{32}P$ labeled DNA probes specific for the aforementioned partial cDNA sequences (see previous page). Preliminary 5 results indicate that IFNR $\alpha\beta$ and CBG mRNA are expressed in livers of GH TM and not in NT littermates.

Additional information on preparation of DIG-labeled probe for Northern blot analysis.

Non-radioactive DIG-labeled probe for Northern blot was 10 constructed by amplification of the target sequence in the first PCR step followed incorporation of digoxigenin-11-UTP or DIG-UTP (Roche) on the antisense strand during the second PCR. In the construction of probe for used in the confirmation of differential gene expression in GH 15 transgenic mice versus non-transgenic mice, fragments from subtractive hybridization that were subcloned into pCR2.1 cloning vector were PCR amplified using primers pCR 2.1A (5' ATTACGCCAAGCTTGGTACCG 3') and pCR IIB (5' CCCTCTAGATGCATGCTC 3'). Incorporation of DIG-UTP is accomplished using primer 20 pCR 2.1A or pCRIIIB in the second PCR step. pBluescript plasmid with the 'full-length' cDNA 45 and cDNA 5 probes, respectively. T3 (5' AATTAACCCTCACTAAAGGG 3') and mKS (5' CCTCGAGGTGACGGTATC 3') primers were used for the first PCR amplification step and mKS primer for the second DIG-UTP 25 incorporation step.

Example 2

A cDNA library was constructed from the liver of growth hormone (GH) transgenic mice. The cDNA that was used in the construction of the cDNA library was prepared from mRNAs, 30 which was obtained from total RNA isolated from the liver of GH transgenic mice. The cDNA prepared was then used to produce the lambda zap (Stratagene) cDNA library. The titer of the amplified library was 10^9 pfu/ml and the recombination efficiency determined to be 75%. Screening of the cDNA 35 library for novel genes was done by probe hybridization of the nitrocellulose plaque lifts. The probe used in the screening was prepared by PCR amplification of gene

fragment, which previously was identified by subtractive hybridization as differentially expressed in GH transgenic mice and not in wild type mice. After screening of approximately 2.5×10^5 plaques, five plaques that hybridized 5 with the probe were purified and then the pBluescript plasmids, which contain the cDNA inserts, were excised out of the lambda zap vector utilizing helper phages following the manufacturer's protocol. The cDNA sequence of the insert was determined by "walking" through the sequence 10 starting with T3 and KS primers complementary to sequences in the plasmid vector.

Clone 5

The sequencing of cDNA for one of the positive clones that hybridize with probe 5 is completed (Table 2A).

15 Using GeneRunner software program, the translational reading frames were determined. The DNA sequence (SEQ ID NO:7) of Clone 5 has several ORFs; the longest, corresponding to 1548 bases, encodes a protein of 515 amino acid residues (SEQ ID NO:8). All ORFs are set forth in 20 Table 3B.

Using BLAST (Basic Local Alignment Search Tool) programs, which are designed to compare DNA and protein sequences available in the database, the DNA and the corresponding protein sequences were found to be novel.

25 Protein motif search utilizing PROSITE database indicate that the protein corresponding to the longest open reading frame in cDNA sequence of Clone 5 possess the following motifs: N-glycosylation, protein kinase C phosphorylation, casein kinase II phosphorylation, and 30 amidation sites. The protein appears to have a signal peptide but no transmembrane region found. Thus, this protein encoded by the longest open reading from in Clone 5 could be cytoplasmic in location.

Clone 45

35 The sequencing of cDNA (SEQ ID NO:9) for one of the positive clones that hybridize with probe 45 is complete

(Table 3A).

Using GeneRunner software program, the translational reading frames were determined. The DNA sequence of Clone 45 has several ORFs; the longest, corresponding to 1029 bases (SEQ ID NO:9) which encodes a protein of 342 amino acids (SEQ ID NO:10). All ORFs are set forth in Table 3B.

Using BLAST (Basic Local Alignment Search Tool) programs, which are designed to compare DNA and protein sequences available in the database, the DNA and the corresponding protein sequences were found to be novel.

Protein motif search utilizing PROSITE database indicates that the protein corresponding to the longest open reading frame in cDNA sequence of Clone 45 possess the following motifs: N-glycosylation, protein kinase C phosphorylation, casein kinase II phosphorylation, and amidation sites, as well as a Myc-type helix-loop-helix dimerization domain. The protein appears to have signal peptide at the N-terminal and transmembrane region close to the N-terminal. This could indicate that the protein encoded by longest open reading frame in Clone 45 is membrane bound and/or secreted.

Significance of the protein motifs found in novel cDNA sequences isolated from the livers of GH transgenic mice:

N-glycosylation: post-translational modification of proteins involving attachment of carbohydrate residues. This modification is seen in secreted and membrane proteins. Glycosylation is associated with lengthening biological life of a protein by decreasing its rate of clearance from the serum. For membrane bound proteins, carbohydrates are usually involved in interaction with other cells or molecules, such as immunoglobulins, cell surface receptors, and proteases.

Phosphorylation sites: site of attachment of phosphate group. Reversible phosphorylation-dephosphorylation of protein is associated with regulation of activity of the protein. Some proteins are activated when phosphorylated and inactivated when unphosphorylated, or vice versa.

N-myristoylation: (usually at the N-terminus) this protein modification involves addition of myristoyl group which is believed to cause some of the attached proteins to be loosely associated with membranes. Some myristoylated 5 proteins are not associated with membranes to any significant extent. Some of myristoylated proteins such as protein kinases and phosphatases have important roles in modulating cellular metabolism.

Amidation: is usually seen on carboxy-terminus of 10 peptide hormones. Enzymes involved in amidation reaction are usually found in secretory granules.

Myc-type, helix-loop-helix (HLH) dimerization domain: HLH dimerization domains are usually present in proteins that interact with DNA. Myc proteins are involved in growth 15 regulation.

References:

Creighton, T.E. 1993. Proteins: Structures and Molecular Properties, 2nd ed. W.H. Freeman and Co., NY, pp. 78-99.

20 Lewin, B. 1994. Gene V. Oxford University Press, NY, pp. 899-902.

Example 3

Assay for using mouse DNAs presence of genes from liver of human patients

25 **Total RNA preparation human liver**

Total RNA will be extracted from liver biopsy using 10 mL RNAStat60 per gram of liver tissue. To 15-20 µg of liver RNA isolates, 1X MOPS, formaldehyde, formamide and ethidium bromide will be added, heat denatured at 60 °C then loaded on 30 a formamide containing denaturing 1% agarose gel. The RNA will then be resolved by electrophoresis at 50V for about 2-2½ h. After electrophoresis, the gel will be washed twice briefly with deionized water; then once with 0.05N NaOH, with 0.1M Tris at pH 7.5, and with 10X SSC at washing times 35 of at least 30 min in each case.

The resolved RNA after electrophoresis will be

transferred onto a nylon membrane by upward gradient adsorption using 10X SSC as transfer buffer. The RNA on the membrane will be UV crosslinked at 120 mJ, after which the RNA blots will be ready for hybridization.

5 **B. Northern Blot Hybridization involving Non-radioactive DIG-labeled probe**

Northern blot hybridization using digoxigenin (DIG)-labeled probe will be conducted to determine whether the genes of interest are present in liver RNA blots. The probes to be used for hybridization will be prepared from pCR2 clones, which contain as inserts the fragments isolated by subtractive hybridization of liver genes from GH mice versus WT mice. The sequence homology of the fragments to that of the human genes range from about 74% to 94%, which were obtained using the default parameters of Blast 2.0 sequence alignment version blastn 2.0.8.

1. Preparation of DIG-labeled probe

The DIG-labelled probe preparation will require PCR amplification of the inserts in pCR2 clones using Taq polymerase as polymerization enzyme and pCR 2.1A and pCR 2B as primers. The conditions for PCR amplification will be 95°C for 2 min.; 55 cycles at three temperature conditions of 95°C for 15 sec., 58°C for 20 sec., and 72 °C for 45 sec.; then 72°C for 7 min. The amplified double-stranded cDNA fragment will undergo a second PCR amplification using a single primer, pCR 2.1A, in the presence of DIG labeled dNTPs to produce a single stranded DIG-labeled PCR product which will serve as the probe for RNA blot hybridization. The concentrations of the DIG labeled probe will be determined by comparing the signals produced by the probe to that of control DIG-labeled DNA upon exposure to radiographic film.

2. RNA Blot Hybridization

The concentration of DIG-labeled probe to be used for hybridization will be 50ng/mL of DIG Easy Hyb solution (Boehringer-Mannheim). Prior to hybridization, the RNA

blots will be prehybridized in DIG Easy Hyb solution at 42 °C for 30-60 min. Following prehybridization, the RNA blots will undergo hybridization using the probes prepared from the different pCR 2 clones. Hybridization will be done at 5 42 °C for at least 8 hours.

Posthybridization washings of the membrane will then be performed at room temperature for 5min using a solution of 2X SSC and 0.1% SDS; and twice at 60 °C for 15 min. using a solution of 0.5X SSC and 0.1%SDS. The RNA blots will then 10 be incubated with DIG antibody, which is conjugated to alkaline phosphatase. This antibody recognizes the DIG labeled hybrids in the RNA blot. CSPD (Boehringer-Mannheim), which is a chemiluminescent substrate for alkaline phosphatase, will be used to achieve detection of 15 the RNA of interest in the blot. The presence of bands that is specific to the liver genes of interest could be diagnostic of liver damage.

Northern Blot Hybridization involving ^{32}P -labeled probe

1. Preparation of ^{32}P -labeled probe

20 The ^{32}P -labeled probe will be prepared by first isolating the cDNA fragments that were inserted into the pCR 2 vector by performing EcoRI restriction enzyme digestion. The fragments will be purified through a Qiaex® agarose gel extraction column (Qiagen). A 25ng of the purified fragment 25 will serve as a template for the production of single-stranded ^{32}P -labeled probe using Random Primed DNA Labeling kit (Boehringer-Mannheim). The unincorporated dNTPs will be separated from the radiolabeled fragments using STE Select D G-25 column. The purified radiolabeled probe will then be 30 quantified to determine the activity of the probe per ug of the DNA template. A good labeling of the template would have a specific activity range of $10^8\text{-}10^9$ cpm/ug of the template DNA.

35 2. RNA Blot hybridization

Prior to hybridization, prehybridization of the RNA blots will be performed by incubating the membrane in

prehybridization solution made up of 50% formamide, 1% SDS, 1M NaCl, and 10% Dextran sulfate for 1 hour at 42 °C. Hybridization of the RNA blot with the ³²P-labeled probes prepared will follow after prehybridization. This will be 5 conducted at 42 °C for at least 8 hours. Washing of the blots will be conducted once with 2X SSC at room temp for 5 min. and then with 2X SSC, 0.1% SDS at 56 °C which could last for about 5 minutes to an hour depending on the intensity of the radioactive signal. Radiographic exposure of the blots 10 will determine whether the genes of interest are present.

Table A

Human Genes (counterpart of Mouse Genes) regulated by Growth Hormone in Liver Tissue

| | Genes | Nucleotide Accession No. | Protein Accession Number |
|----|---------------------------------------|---|---|
| 5 | Human alpha-fetoprotein | NM_001134 V01514 | NP_001125 CAA24758 |
| | Human Fetuin (A2HS) | M16961 | WOHU AAA51683 P02765 S04467 |
| | Human 3-beta-hydroxysteroid Type 1 | M27137 | AAA36015 dehydrogenase |
| 10 | Human Rab8 Interacting protein-like 1 | NM_003618 | NP_003609 |
| | Human Paraoxonase-3 | L48516 | AAC41996 |
| | Human IFN alpha/Beta Receptor | A32391 | CAA02098 |
| | Human GHR | AAA52555 | M28458 |
| | Human Cytochrome P450 IIIA | NM_000777 X12387 M18907 J04449 NM_000765 M14096 NM_000776 | NM_000768 CAA30944 AAA35745 AAA35747 NP_000756 AAA35744 NM_000767 |
| 15 | Human proteasome z-subunit | D38048 | BAA07238 |
| | Human Corticosteroid binding Globulin | NM_001756 | NP_001747 |
| | Human Coagulation Factor V | M16967 NM_000130 | AAA52424 NP_000121 |
| 20 | Rat S-2-hydroxyacid oxidase | X67156 | CAA47629 |

Table B: Result of Blast Search

| <u>Clone</u> | <u>Closest Match</u> | <u>Identities</u> |
|--------------|--|-------------------|
| 2 | Mouse alpha fetoprotein M16111 | 77/78 |
| 5 6 | Mouse fetuin AJ002146 S96534 | 70/70 78/78 |
| 7 | mouse 3-beta hydroxysteroid dehydrogenase | |
| 10 | M77015 | 78/78 |
| 13 | mouse rab8-interacting protein U50595 | 66/66 |
| 14 | mouse paraoxonase-3 | |
| 15 | L76193 | 64/64 |
| 21 | rat S2 hydroxyacid oxidase X67156 | 58/65 |
| 20 | mouse interferon α/β receptor M89641 U06244 | 78/79 78/79 |
| 29 | mouse low MW GH receptor | |
| 25 | M31680 M33324 | 59/61 59/61 |
| 20 | mouse cytochrome P450 IIIA X60452 | 62/64 |
| 30 36 | same | 69/78 |
| 39 | mouse cytochrome P450 III A D26137 | 75/81 |
| 35 37 | mouse proteazome Z subunit D83585 | 77/78 |
| 34 | corticosteroid-binding protein X70533 | 46/46 |
| 40 35 | same X70533 | 37/37 |

42

| | | |
|------|-------------------------------|---------|
| 52 | same X70533 | 121/123 |
| 49 | same X70533 | 46/46 |
| 5 56 | mouse coagulation factor V | 104/106 |

Table 1 shows the sequence of each clone, and its BLASTN alignment to the known mouse (or rat) gene as found in a sequence databank, to which it appears to be most closely related. The known genes are as follows:

- 5 (A) mouse alpha-fetoprotein (WLAC #2)
- (B) mouse fetuin (WLAC #6)
- (C) mouse 3-β-hydroxysteroid (WLAC #7)
- (D) mouse rab8 interacting protein (WLAC #13)
- (E) mouse paraoxonase-3 (WLAC #14)
- 10 (F) rat S-2-hydroxyacid oxidase (WLAC #21)
- (G) mouse interferon α/β receptor (WLAC #26)
- (H) mouse growth hormone receptor (WLAC #29)
- (I) mouse cytochrome P450IIIA (WLAC #20, #36)
- (J) mouse cytochrome P450 (WLAC #39)
- 15 (K) mouse proteasome z-subunit (WLAC#37)
- (L) mouse corticosteroid binding globulin ((WLAC #3, 34, 35, 52)
- (M) mouse coagulation factor V (WLAC #56)

Table 2 (A) full-length single stranded nucleotide sequence
20 of clone 5 and (B) ORFs 1-16 corresponding to clone 5.

Table 3 (A) full-length single stranded nucleotide sequence
of clone 45 (B) ORFs 1-9 corresponding to clone 45.

Table 1**WLAC #2 -fetoprotein****WLAC #2 SEQUENCE**

TCCTAGGCTTCTGCAGCCTCCACGAGAGTTGGGTTGACACCTGAGGTGCTTCTGGGTAGCGAA

5 CTAGAATGGCATTGGATCCATATTCTCCACCGCCCTCC

Sequence 1 1c11seq_1 WLAC #2 Length 109 from:1 to = 109**Sequence 2 gi|191764 Mouse alpha-fetoprotein mRNA, partial cds.**
Length 1254 from:1 to = 1254

10 NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 187 bits (97), Expect 2e-46

Identities = 99/100 (99%), Positives = 99/100 (99%)

Aligned query 1-100 to subject 1071-972.

15 **WLAC #3, 34, 35, 52 Corticosteroid Binding Globulin****WLAC #3 SEQUENCE**CATGGTGGGAGCCAGGTCTCGTGAGAACTTGAATCCTCATCAGTGACAGCC
TGGGTGGTCCAGAGGCCACTGGTGCAGAGCCAGAAGAGACAGGTATAACAGGG
CGAGCGACATTGTTTGG20 **Sequence 1 1c11seq_1 WLAC#3 Length 124 from:1 to = 124****Sequence 2 gi|298114 M. musculus mRNA for corticosteroid-binding globulin**
Length 1462 from:1 to = 1462

Score = 217 bits (113), Expect = 1e-55

Identities = 122/124 (98%), Positives = 122/224 (98%), Gaps = 1/124 (0%)

25 Aligned query 1-124 to subject 169-47.

WLAC #34 SEQUENCE

GGCAGCAGGCAGCACATTCCCTTCATCCAGTTGCAGCATGGCCTTGCTGGCGCTACCGCCCTCCGCACCACGCCCTAACGCCGAATTCTGCCATACTATCCATCACACTGG

5 **Sequence 1** 1c11seq_1 **WLAC #34** **Length** 110 from:1 to = 110

Sequence 2 gi 298114 M. musculus mRNA for corticosteroid-binding globulin
Length 1462 from:1 to = 1462

Score = 89.1 bits (46), Expect = 8e-17

Identities = 46/46 (100%), Positives = 46/46 (100%)

10 Aligned query 1-46 to subject 116-1071.

WLAC #35 SEQUENCE

GCAACTGGATGAAGGGAATGTGCTGCCCTGCTGCCACCAATGGAAATCCTGTACCGCCCTCCGACCACGCCCTAACGCCGAATTCTGCAGTCTAT

Sequence 1 1c11seq_1 **WLAC #35** **Length** 95 from: 1 to = 95

15 **Sequence 2** gi 298114 M. musculus mRNA for corticosteroid-binding globulin
Length 1462 from:1 to = 1462

Score = 85.3 bits (44), Expect = 9e-16

Identities = 50/53 (94%), Positives = 50/53 (94%)

Aligned query 1-53 to subject 1083-1135.

20 WLAC #52 SEQUENCE

GCCTGACTGGACCATCATGGGCACCTTCACTGTGCTTGTCTCATTCACATAGAA GTCCCTCTCTCAGTATTTCTGGGCTGAAGGGAAGTTCCATATTCCCTTGAGGAAGAGTAGTTGAT

Sequence 1 1c11seq_1 **WLAC #52** **Length** 121 from:1 to = 121

25 **Sequence 2** gi 298114 M. musculus mRNA for corticosteroid-binding globulin
Length 1462 from:1 to = 1462

Score = 206 bits (107), Expect = 4e-52

Identities = 121/123 (98%), Positives = 121/123 (98%), Gaps = 2/123 (1%)

Aligned query 1-121 to subject 747-625.

WLAC #6 Fetuin (AHSG)

5 WLAC #6 SEQUENCE

GGGAGAGGCACATTTGAGCCCGGGAAATCTCACCACTTGGGGTAGGTTCC
ATTATTCTGTGTGTTGAAGCAGCCAGGGCAGTGTGAC

Sequence 1 1c11seq 1 **WLAC #6** **Length 91 from:1 to = 91**

Sequence 2 gi 2546994 Mus musculus fe_ (Ahsg) gene, complete cds.

Length 8946 from:1 to = 8496

Score = 117 bits (61), Expect = 1e-25

Identities = 78/84 (92%), Positives = 78/84 (92%), Gaps = 1/84 (1%)

Aligned query 9-91 to subject 4638-4555.

WLAC #7 3--Hydroxysteroid

15 WLAC #7 SEQUENCE

GGGTCACTGAGCTGGCAAGGCTTGGTACTTGATTAAGGCACTAAATTGGCCT
CTGTGTCAAAAGAAGGCAACAGCACCTGTGTTGCTTTATCCTTAAGT

Sequence 1 1c11seq_1 WLAC #7

Length 103 from:1 to = 103

Sequence 2 gi 194006 Mouse 3-beta-hydroxysteroid dehydrogenase/delta-5-delta-4
isomerase mRNA sequence

Length 1533 from:1 to = 1533

Score = 198 bits (103), Expect = 7e-50

Identities = 103/103 9100%), Positives = 103/103 (100%)

Aligned query 1-103 to subject 1325-1223.

WLAC #13 rab8 Interacting Protein

WLAC #13 SEQUENCE

GGAAAGATCCA ACTGATAACCCGGGGCACACAGCAACCTCTACATCCTCAC
GGGTCA CCAGAGCAGCTACTGAGCTATCTCCCGATGACGCCAAGCCCTCGGC
5 CTC**Sequence 1** 1cl1seq_1 **WLAC #13** **Length** 108 from:1 to = 108**Sequence 2** gi1330327 Mus musculus Rab8-interacting protein mRNA, complete
cds.

Length 2466 from:1 to 2466

10 Score = 127 bits (66), Expect = 2e-28
Identities = 66/66 (100%), Positives = 66/66 (100%)
Aligned query 9-74 to subject 2401-2466.

WLAC #14 Paraoxonase-3

WLAC #14 SEQUENCE

15 GGCATAGAACTGCTCTGGCCCAAGAACCAATGTCATTACACTTTGAGAA
GTTCATGTTTGAGATTTCAGGTGGATGAGAGAGAGCGTTGTTCTTCAAA**Sequence 1** 1cl1seq_1 **WLAC #14** **Length** 107 from: 1 to = 107**Sequence 2** gi 1333639 Mus musculus paraoxonase-3(pon3_ mRNA, complete cds.
Length 1121 from:1 to = 1121

20 Score = 162 bits (84), Expect = 7c-39
Identities = 101/107 (94%), Positives = 101/107 (94%), Gaps = 2/107 (1%)
Aligned query 1-107 to subject 537-433.

WLAC #20, #36 Cytochrome P450IIIA

WLAC #20 SEQUENCE

25 GGAGCATGAGTTCCCTCAAGGAGTTCTGCTGAGTTCTCAGAAAGGCAGTGT

CTAAGAACATCAGATATG

Sequence 1 1cl1seq_1 **WLAC #20** **Length** 71 from:1 to = 71

Sequence 2 gi 50534 M. musculus mRNA for cytochrome P-450IIIA
Length 1690 from:1 to =1690

- 5 Score = 112 bits (58), Expect = 4e-24
Identities = 62/64 (96%), Positives = 62/64 (96%)
Aligned query 1-64 to subject 1581-1644.

WLAC #36 SEQUENCE

AAAGGATCACAAAAGTCAACTATTAAAATCCCTTGGCTTCCTCCACAAAGGG
10 ATCCTCTAAACTTGTTGAGGGAATCCACATTCACTCCAAA

Sequence 1 1cl1seq_1 **WLAC #36** **Length** 93 from: 1 to = 93

Sequence 2 gi 50534 M. musculus mRNA for cytochrome P-450111A
Length 1690 from:1 to = 1690

- Score = 94.9 bits (49), Expect = 1e-18
15 Identities = 80/93 (86%), Positives = 80/93 (86%), Gaps = 1/93 (1%)
Aligned query 1-93 to subject 730-639.

WLAC #21 S-2-Hydroxy acid oxidase

WLAC #21 SEQUENCE

AACCCAAGTTCCTACAGCATCTTGAGCTGTTGATCTCACTCTTCGTTCTAT
20 TGGAGAAACTACCGGCCAGCAATGTCTTG

Sequence 1 1cl1seq_1 **WLAC #21** **Length** 85 from:1 to = 85

Sequence 2 gi 311832 R. norvegicus mRNA for (s)-2-hydroxy acid oxidase
Length 1648 from:1 to = 1648

Score = 79.5 bits (41), Expect = 4e-14

Identities = 75/87 (86%), Positives = 75/87 (86%), Gaps = 2/87 (2%)

Aligned query 1-85 to subject 125-211.

WLAC #26 Interferon α/β Receptor

WLAC #26 SEQUENCE

- 5 GGCCACACTGAGATCTAAACAACGCCAGCTCCTCCAGTTAGTGTCCCTTCTC
CATGGTTCAGTGACTTCTGGTCAGAAG

Sequence 1 1c11seq_1 **WLAC #26** **Length 82 from:1 to = 82**

Sequence 2 gi 194111 Mus musculus interferon alpha/beta receptor (IFNAR) mRNA, complete cds.

Length 3894 from:1 to = 3894

Score = 144 bits (75), Expect = 8e-34

Identities = 82/83 (98%), Positives = 82/83 (98%), Gaps = 1/83 (1%)

Aligned query 1-82 to subject 2222-2140.

WLAC #29 GHR

15 WLAC #29 SEQUENCE

TTGCTGGACCCGGGGTCGTTCACTGTTGACCGAAATAGTGCAACCTGATCC
ACCCATTGGCCTAAGTGGACTTTACTAAA

Sequence 1 1c11seq 1 **WLAC #29** **Length 82 from:1 to = 82**

Sequence 2 gi 193508 Mouse high molecular weight growth hormone receptor/binding protein, complete cds

Length 2288 from:1 to = 2288

Score = 94.9 bits (49), Expect = 9e-19

Identities = 63/65 (96%), Positives = 63/65 (96%), Gaps = 2/65 (3%)

Aligned query 19-82 to subject 541-604.

- ### 25 WLAC #37 Proteasome z-subunit

WLAC #37 SEQUENCE

TGTCTCACCGAGAAAGTTACCCCTCTGGAGATTGAGGTGCTAGAAGAGACTG
TTCAGACAATGGATACTTCGTAATGGTG

Sequence 1 1cl1seq_1 **WLAC #37** **Length** 81 from:1 to = 81

5 **Sequence 2** gi:1632754 Mouse mRNA proteasome Z subunit, complete cds.
Length 969 from:1 to = 969

Score = 142 bits (74), Expect = 3e-33

Identities = 74/74 (100%), Positives = 74/74 (100%)

Aligned query 1-74 to subject 763-836.

10 **WLAC #56 Coagulation Factor V**

WLAC #56 SEQUENCE

TGTGGCTTCTGAAAAGGGTAGTTATGAAATAATAGCAGCAAATGGCGAAGAC
ACAGATGTGGATAAGCTGACCAACAGTACCTCAAAATCAGAATATCACAGTA
CCGCCCTCCGCACCACGCCCTAACGCCGAATTCTCGAGAT

Table 2A
Full-length nucleotide sequence of Clone 5
(5' → 3' direction of the + strand)

AAGACCCGCCATGTCTCTGCTGGCTACTGTACTGCTGCTCTGGGGG
5 TTCACTCTGGGCCAGGAAATACTCTAACATGCTCGATTCTGGCAGTG
AACCTAAACTATGGGCAGAGCCTCAGTCCCTGCTGGAACCCTGGG
CAAACCTGACCCTGGTGTGCAGTTGATTGCCGACTAAGGTCTT
CGAGCTGATCCAGAACGGGTGGTCCCTGAGTCAAGTCCGACTTGA
GACACAGGTGCTGTCAACCGCTTTCCCTGGGGGCCATTACAAGT
10 AACAACAGTGGCATCTACCGCTGCAGATGTGGCGTGGAACCCCCCT
GTTGACATTCACCTGCCAGCACTGAACAAAGTGGACCATGCTAACG
AATGCTGTGGAGGTGACAGGGAAAGAGGCCCTGCCTCGGCCCTTG
GCTCATGCTGATCCAGTCAGTGGATCACACCTGGTGGCCTGCCTG
TATACGTGATGTGCCAGGTTGCAATGCCGGGTGTGACCTACCTGCT
15 GAGGCAGGAAGGAGTGGATGGCGTCCAGAACCTGATGTCCAGC
ACAAGGAAACAGCTGGCTTTTAATCTACAAAGCCTGGCAACTACA
GCTGCAGCTACCTAACCCATGCAGCAGGTGAACCCTCTGAGCCA
GTGATATTGTGACCATCAAGATGTATGCCTCACAGGCTCCACCCAC
TCTGTGTTGATGGGAAACTACCTAACCTGATCTACCCCCAGAAGACA
20 TATGAGACCCCTGCCTGCAAAGCTCCTCGGAATGCAGCTGAATTCC
AACTCAGGCAAGGAGGGAAAGGTGCTGAAAATTGATGGGTTAGCC
CCACCAAGAGATGCTATCCTGTACTATGTGAACCTGAAGGAACCTGG
ATAACCCAGGTCTTACACCTGCCGCTACCGGATGCACAAATACAT
GCACGTTGGTCAGAGGACAGCAAGCCCCTAGAGTTAATGTGGAG
25 TGATGAGACTCTACAAGCTCCGGTACTTACTGCAGAGCCATCGAG
TAGGGACCTTGAGCCTGGTTCAACGGTGCAGCTCGATGTACTGCA
CCCGTATCCGGCCTGCGCTTGGCCTGCAACGCCAGGGCAAACCG
GAATTAGTTGTGGTGCAAATGCTGAATTGCTCTGGGACCGAAGCA
GTCTTGAGCTGCACAATATCTAACAAATAGACTCTGGAAACTACA
30 GCTGTATCTACATGGAACAGGCACCGCCATTCTCAGGATCTTCTTC
CACTGAGCCCGTGGAGCTGCGGGTGAATGGGCCACCACCAAGCC

AAGGCTGGAAGCTCTGTGGAAAAGCACAGTACATCTGGGTCAAGGA
AGCCATCTTCGATGCCACGGCCATGTGCCTAGGGTCAGCATGGA
GCTGGTACGTGAGGGCTTAAAACACCCCTCGCGTGGCCTCCAC
AAGAAGCACCTCAGCTTACCTGAAGCTGCTTCGTCGGTCCCCAA
5 CATGCAGGCAACTACAGCTGCCGCTATACGGCCCTGCCGCCITC
ACATTGAATCAGGGATCAGCGACCCCTGTGGAGGTTATAGTAGAA
GGTTAGGCTCCTGAGCTGTGTTGAGGTTTGGGTTCTTAATAT
TTCCAGAGCTGTACACTGGCTAATTGCTTCACCAAGGTCAAGTGTGG
AAAGGCCCTGTGGCAACTTGCTGAGTCAATGAAGCCATTCTTGT
10 CTAGGCCGCTAATGTGGCTGCAGACACAAAAAAAGTGTCTTGGG
AAGGGTTCAAGACAGGTATAATACCCATTCTCTCAATGTAAGAT
AACTTCATTTCTGGACTTAATAAGGTCAAGTAAAAACCCGTT
TAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
15 AAAAAAAAAAAAAAAAAAAAAAGCTTGA
 CAAAA

Table 2B
Open Reading Frames found in Clone 5

MSLLATVLLLWGFTLGPGLMLDSGSEPKLWAEPQSLLEPWANLTL
VCAVDLPTKVFELIQNGWFLSQVRLETQVLSYRFSLGAITSNNSGIYR
5 CRCGVEPPVDIHLPALNKWTMLSNAVEVTGKEPLPRPLAHADPVDWI
TPGGLPVYVMCQVAMRGVTYLLRQEGVDGVQKPDVQHKGTAGFLI
YKPGNYSCSYLTHAAGEPSEPSDIVTIKMYASQAPTLCLMGNYLMY
PQKTYETLACKAPRNAAEFQLRQGGKVLKIHGFSPTRDAILYVNLK
ELDNPGPFTCRYRMHKYMHVWSEDSKPVELMWSDETLQAPVLTAEP
10 SSRDLEPGSTVQLRCTAPVSGLRFGLQRQGKPELVVVQMLNSSGTEA
VFELHNISTIDSGNYSCIYMEQAPPFGSSSEPVELRVNGPPPKRLEA
LWKSTVHLGQEAIFRCHGHVPRVSMELVREGFKTPFAVASTRSTSAY
LKLLFVGPQHAGNYSCRYTALPPFTFESGISDPVEVIVEG@
(SEQ ID NO:8)

15 MWLQTQKKCSWEGFKTGIIPIILLNVR#

MYCTRIRPALWPATPGQTGISCGANAEFVWDRSSL&

MQATTAAAIRPCRPSHLNQGSATLWRL@

MRLYKLRYLLQSHRVGTLSLVQRCSDVLHPYPACALACNARANRN
@

20 MGHHPSQGWKLKGKAQYTWVRKPSFDATAMCLGSAWSWYVRALK
HPSRWPPQEAPQLT&

MKLSYIEKNGYYTCLEPFPRTLFLCLQPH@

MSSGGRSLGSFPSNTEWVEPVRHTS&

MGIIPVLNPSQEHFFCVCSHISGLDKEMASLTQQVATGPFHTDLGEAIS

QCTALEILRTQNLKHSSGEPNLLL#

MAVPVPCRYSCSFQSLLRYCAAQRLLRSQTNSAFAPQLIPVCPGVAG
QSAGRIRVQYIEAAPLNQAQGPYSMALQ#

MNFQHLPSLPELEFSCIPRSFLAGKGLICLLGVDH@

5 MAVASKDGFLTQMYCAFPQSFQPWLGGWWPIHPQLHGLTGRRS&

KTRHVSAGYCTAALGVHSGPRKYSNARFWQ&

FCQAFFFFFFFFF¹²FFFFKRVFT&

FVKLFFFFFFF¹²FFFFLNGFLLDLY#

LSSFFFFFFF¹²FFFF#

10

Note: First 12 sequences are ORF starting from met to stop codon, the next four sequences were also identified as ORFs from the beginning of the sequence to the stop codon. ORF analysis conducted using GeneRunner version 3.05 software by Hastings Software, Inc.

15 @ = TAG, & = TGA, # = TAA

Table 3A**Full-length nucleotide sequence of Clone 45****(5' → 3' direction of the + strand)**

5 GCTGAAC TGAAG ACCCGCCATGTCTCTGCTGGCTACTGTACTG
 CTGCTCTGGGTTTCACTCTGGACCCAGCAACGGATGCAGCCA
 CCTGTACATTCAAGGATGCCATAAAAAACAATTCTTGCCCAG
 GCCCTGGATTTGCCTTATCCTGTGCCTGGATCATACCTGGC
 CTGATCACGTCCGTGTTGTGCCTGGGGAGAGTGAAGGGGCA
10 GCCTTCCTGCTGAGGCGGGAAAGGAGATGATGACTTCCTAGAG
 GTAGCTGAAAATACCACTGTTTCGGGGATGAAACTCAGGCAG
 GATACAGGGAACAAAGCCATGTTCGAGTCTATCAACCAGGCAA
 CTACAGCTGCAGCTATCAAACTCACGGAGAATGTACCTCCTCT
 ACGCCAGTAGGATTGTGACCATCAAGAAGTTGCCAACAGTGGAGCC
15 CGCCACCCCTGCTGACCTCCTCAGAAAGTTCCACAGTGGAGCC
 ACCCCACATGGCCCGTATGACCCCTCTGTTCCACTTTCTG
 AACGACGTTGAATTCAGCTGAGGCAGGGAAAGCGTGAGATG
 AAGGTCTTATGTTCAGCACCAAGCCAGAGCAAGTCAACTTCT
 ATCTGAAATTGTCAGACATGGGTGACCAAGAGCCCCCTCACCTG
20 CCGCTACCGTCTAACGCAACATGACAGCTTGGTCGGAAGACAGT
 GAGCCCCTAGAGCTAATGTGGAGCGACGAAAGACTACCAGCA
 CCAGTGTGACTGCAGAGCCATCGACGAATCAGAGCTTGAGC
 CGGGTTCGACGGTGCAGTTCGATGTACCGCACCCAGGCTG
 GCCTACGCTTGTAGTCTGGCCTGCGCTTGGCCTGCATACCGA
25 AGACTTGTATGAGCGCAGCCTGATCCAGATACTGAAGTCTTCT
 GGTCATGAAACTGTATTCCAGCTGCAAAACCTCTCAGCCGCAG
 ATTCTGCCAGCTACAGCTGCATCTACTGAACGAAACTCCACC
 CTTCTCTGGATCTGCTCCAGCAACCTGTGCCTCTGATGGTG
 GACGGATCCTACGAGTACTGAACCTATAGTAAACTGGAGCT
30 GCATTTGTGGGTCCCACATACAGGAAACTATACTGCCGT
 TATACCTCCTGGCAGCCTGAGCCGTCACTCAGAGCCCAGCA
 ACTCCGTGGAGCTCCTAGTGGAAAGGTATGGCAGTGGTTGGGT

TTTGCCTCTGATCTTGTGGACTATGCATTCAAGTTAATTGTG
TGATCTAGCCTGGTATTCAAAGGCCCGTGGCAGTTGCTGAG
TCAAGTCACCTACTACTTGTCTGGGAAACTGAAGTAGCTGCA
GACACAGGACCAAACATTGTTCTGGAAAGAGCAGAAGACAG
5 ACGGGCAGAACTCCTATTCTCCTGCTGCAAGATGTATTC
TCAAACCTCCACTTAATAAAGATCAAAAAAAAAAAAAA

Table 3B**Open Reading Frames for Clone 45**

MSLLATVLLLWGFTLDPATDAATCTFKDAIKNNSLPRPWILPYPV
PWIIPGLITSVLCLGRVKGAFAFLRREGDDDFLEVAENTTVFGDE
5 TQAGYREQAMFRVYQPGNYSCSYQTHGECTSSTPSRIVTIKKFAK
PPPPLTSSESSTVEPPHMARMTLLCSTFLNDVEFQLRQGKREMK
VLMFSTSPEQVNFKLSDMGDQSPFTCRYRLSNMTAWSEDSEP
VELMWSDERLPAPVLTAEPSTNQSFEPEGSTVQFRCTAPKAGLRFE
SGLRFGLHTEDLYERSLIQILKSSGHETVQLQNLSAADSASYSCI
10 YTELKPPFSGSAPSNLVPLMVDGSYEV&
(SEQ ID NO:10)

MKLRQDTGNKPCFESINRATTAAIKLTENVPLRPVGL&

MTRRLQYLDQAALIQVFGMQAKAQARLKA@

MWGGSTVELSEEVSRGGGLANFLMVTILLGVEEVHSP&

15 MALQSTLVVVFRRSTLALRAHCLPTKLSCLDGSGR&

MHSPTKIKRQNPTTAIPSTRSSTELLGSEWTGSGCQE#

AELKTRHVSAGYCTAALGFHSGPSNGCSHLYIQQGCHKKQFLAQA
LDFALSCALDHTWPDHVRRVPGESERGSLPAEAGRR&

20 FFFLIFIKWREFEGNTCSRKNRSSARLSSALSKNVVWSCVCSYFS
FPDKVVGDLTQQTATGPLNTRLDHTIN&

MASLNQVAAASVAGSRVKPQSSSTVASRDMAGLQFS

Note: First six sequences are ORFs starting from met to stop codon, the next two sequences were also identified as ORFs from the beginning of the sequence to the stop codon and the last ORF is the sequence starting from met to the end of the sequence. ORF analysis conducted using

- 5 **GeneRunner version 3.05 software by Hastings Software, Inc.**

CLAIMS

1. A method of diagnosing abnormal levels of growth hormone (GH) activity in the liver, or of predicting a change in the condition of the liver in response to abnormal levels of GH activity therein, which comprises
 - (A) obtaining a sample of one or more liver cells,
 - (B) assaying messenger RNA of said sample, or complementary DNA reverse transcribed from said messenger RNA, to determine the level of transcriptional activity of one or more of the following genes in said cell:
 - alpha-fetoprotein gene
 - fetuin gene
 - 3-β-hydroxysteroid gene
 - rab8-interacting protein gene
- 10 15 paraoxonase-3 gene
- interferon α/β receptor gene
- proteasome z-subunit gene
- corticosteroid binding globulin gene
- growth hormone receptor gene
- 20 cytochrome P450IIIA gene
- cytochrome P450 gene
- coagulation factor V gene
- S-2 hydroxyacid oxidase gene
- human gene at least 50% identical to SEQ ID NO:7
- 25 human gene corresponding to at least 50% identical to SEQ ID NO:9
- and
 - (C) correlating the level of activity with the level of GH activity in the liver or the expected change in the condition of the liver as a result of such GH activity.
- 30 2. A method of diagnosing abnormal levels of growth hormone (GH) activity in the liver, or of predicting a change in the condition of the liver in response to abnormal levels of GH activity therein, which comprises
 - (A) obtaining a sample from said patient, where said sample is expected to contain protein produced by the liver,
 - (B) assaying the protein in said sample to determine the level of expression of one or more of the following

- proteins:
- alpha-fetoprotein
- fetuin
- 3- β -hydroxysteroid
- 5 rab8-interacting protein
- paraoxonase-3
- interferon α/β receptor
- proteasome z-subunit
- corticosteroid binding globulin
- 10 growth hormone receptor
- cytochrome P450IIIA
- cytochrome P450
- coagulation factor V
- and
- 15 S-2 hydroxyoxidase protein encoded by a gene corresponding to clone 5 in mice human protein at least 50% identical to SEQ ID NO:8, human protein at least 50% identical to SEQ ID NO:9,
- (C) correlating the level of expression with the level
20 of GH activity in the liver or the expected change in the condition of the liver as a result of such GH activity.
3. The method of claim 1 or 2 where the level of transcriptional activity of the alpha-fetoprotein gene or expression of alpha-fetoprotein is determined.
- 25 4. The method of claim 1 or 2 where the level of transcriptional activity of the fetuin gene or expression of fetuin is determined.
5. The method of claim 1 or 2 where the level of transcriptional activity of the 3- β -hydroxysteroid gene or
30 expression of 3- β -hydroxysteroid is determined.
6. The method of claim 1 or 2 where the level of transcriptional activity of the rab8-interacting protein gene or expression of rab8-interacting protein is determined.
- 35 7. The method of claim 1 or 2 where the level of transcriptional activity of the paraoxonase-3 gene or expression of paraoxonase-3 is determined.
8. The method of claim 1 or 2 where the level of

transcriptional activity of the interferon α/β receptor gene or expression of interferon α/β receptor is determined.

9. The method of claim 1 or 2 where the level of transcriptional activity of the proteasome 2-subunit gene or 5 expression of proteasome 2-subunit is determined.

10. The method of claim 1 or 2 where the level of transcriptional activity of the corticosteroid binding globulin gene or expression of corticosteroid binding globulin is determined.

11. The method of claim 1 or 2 where the level of transcriptional activity of the cytochrome P450IIA gene or expression of cytochrome P450IIA is determined.

12. The method of claim 1 or 2 where the level of transcriptional activity of the cytochrome P450 in gene or 15 expression of cytochrome P450 is determined.

13. The method of claim 1 or 2 where the level of transcriptional activity of the coagulation factor v gene or expression of coagulation factor v is determined.

14. The method of claim 1 or 2 where the level of 20 transcriptional activity of the S-2 hydroxyacid oxidase gene or expression of hydroxyacid oxidase is determined.

15. The method of claim 1 or 2 where the level of transcriptional activity of the gene corresponding to clone 5 in mice gene or expression of gene corresponding to clone 25 5 in mice is determined.

16. The method of claim 1 or 2 where the level of transcriptional activity of the gene corresponding to clone 45 in mice gene or expression of gene corresponding to clone 45 in mice is determined.

30 17. A transgenic nonhuman mammal which exhibits, or has a propensity to develop, a liver pathology said animal comprise a transgene encoding a human protein selected from the group consisting of

alpha-fetoprotein

35 fetuin

rab8-interacting protein

paraoxonase-3

interferon α/β receptor

proteasome z-subunit

corticosteroid binding globulin

growth hormone receptor

cytochrome P450IIIA

5 cytochrome P450

coagulation factor V

said protein being expressed at levels sufficient for said mammal to exhibit, or have a propensity to develop, a liver pathology.

10 18. The mammal of claim 17 which is a mouse, rat or rabbit.

19. The mammal of claim 17 or 18 where expression of said transgene is controlled by a liver-specific promoter.

20 20. An assay for drugs which inhibit the development
15 of, or treat a liver pathology which comprises administering the drug to the mammal of any of claims 17-19.

21. A method of preventing or treating a liver pathology in a subject which comprises administering to the subject a pharmaceutically effective amount of a drug which
20 inhibits the expression or activity of a protein selected from the group consisting of

alpha-fetoprotein

fetuin

rab8-interacting protein

25 paraoxonase-3

interferon α/β receptor

proteasome z-subunit

corticosteroid binding globulin

growth hormone receptor

30 cytochrome P450IIIA

cytochrome P450

coagulation factor V .

22. Use of a drug which inhibits the expression or activity of a protein selected from the group consisting of
35 alpha-fetoprotein

fetuin

rab8-interacting protein

paraoxonase-3

interferon α/β receptor
proteasome z-subunit
corticosteroid binding globulin
growth hormone receptor
5 cytochrome P450IIIA
cytochrome P450
coagulation factor V
in the manufacture of a composition for the prevention or
treatment of liver pathology.

first strand cDNA synthesis is 5' TTTGTACAAGCTT 3' (SEQ ID NO:6) which binds to polyA tail of the mRNA. This primer introduces a unique restriction site Rsa 1 downstream of polyA tail. The second strand cDNA synthesis involves the 5 use of an enzyme cocktail composed of RNase H, DNA polymerase and ligase enzymes.

Once the double-stranded cDNAs from bGH TM (tester) and WT (driver) were prepared, these two cDNA populations were subjected to Rsal digestion to produce shorter, blunt ended 10 fragments. The tester was divided into two halves and each half was then ligated with different adaptors, adaptor 1 and adaptor 2R. These two adaptors have stretches of identical sequences (in bold characters) which serve as sites for binding of PCR primer 1 during the PCR amplification:

15 Adaptor 1: 5'-**CTAATACGACTCACTATA**GGGCTCGAGC~~GGCCGCCGGCAGGT~~-3'
(SEQ ID NO:1)

3'-GGCCCGTCCA-5'

(SEQ ID NO:2)

20 Adaptor 2R: 5'-**CTAATACGACTCACTATA**GGGCAGCGTGGTCGC~~GGCCGAGGT~~-3'
(SEQ ID NO:3)

3'-GCCGGCTCCA-5'

(SEQ ID NO:4)

Since only one end of the adaptors is phosphorylated, ligation of adaptors to tester cDNAs can occur only at the 25 5' ends of the cDNAs.

Isolation of differentially expressed genes from GH TM (tester) is achieved by performing two hybridization steps. The first hybridization step involved mixing each of the adapter ligated testers with excess of drivers. This 30 resulted in annealing of identical ss cDNA fragments common to both the tester and driver. Differentially expressed sequences from GH TM that did not form hybrids with the driver sequences underwent a second hybridization step. This step involved mixing two reaction products from the 35 first hybridization in the presence of more drive cDNA. This resulted in the formation of new hybrids between adaptor ligated ss cDNAs from GH TM. After fill in of the ends of these new hybrids using 50X Advantage cDNA polymerase mix (Clontech), primer sites for PCR primer 1 (5'-